Molecular Diagnosis of Pyrethroid Resistance in Mexican Strains of *Boophilus microplus* (Acari: Ixodidae)

FELIX D. GUERRERO,[1, 2] ANDREW Y. LI,[1] AND RUBEN HERNANDEZ[3]

J. Med. Entomol. 39(5): 770–776 (2002)

ABSTRACT Polymerase chain reaction (PCR) diagnostic assays were used to identify possible resistance-associated roles of two amino acid substitutions found in pyrethroid resistance-associated genes of *Boophilus microplus* (Canestrini). Individual larvae from the San Felipe target site resistant strain and the Coatzacoalcos (Cz) metabolic resistant strain were separated into resistant and susceptible groups by larval packet bioassays and analyzed by PCR. A Phe → Ile amino acid mutation in the sodium channel gene S6 transmembrane segment of domain III was found to have a close association with survival of acaricide treatments containing as high as 30% permethrin. As the permethrin dose was increased, an increase was seen in the proportion of surviving larvae that possessed two mutated sodium channel alleles. An Asp → Asn amino acid substitution, originally found in high allele frequency in alleles of the CzEst9 esterase of the Cz strain, appeared to provide some resistance to permethrin. However, the presence of the mutation did not associate with resistance in the dose–response fashion seen with the sodium channel amino acid mutation. Resistance provided by CzEst9 might be more dependent on concentration of CzEst9 more so than the presence of a mutated allele.

KEY WORDS *Boophilus microplus*, polymerase chain reaction, resistance mechanism, diagnostic, sodium channel, mutation

THE SOUTHERN CATTLE tick, *Boophilus microplus* (Canestrini), is a major problem to cattle producers in many parts of the world because of direct effects of infestation and the diseases these ticks transmit. The tick was eradicated from the United States by an intensive 55-yr USDA program (Graham and Hourrigan 1977) and the United States remains free of *B. microplus* through the efforts of a USDA-APHIS/VS quarantine program established at the border of the United States and Mexico. Additionally, the control of ticks in Mexico is critical to the maintenance of the United States *Boophilus*-free status. The synthetic pyrethroids have played an important role in controlling populations of *B. microplus* in Mexico. However, pyrethroid resistant tick populations were reported in several regions of Mexico only a few years after pyrethroids were approved for use in the *Boophilus* control program in Mexico (Fragoso et al. 1995).

Jamroz et al. (2000) used various biochemical and molecular assays to determine the probable resistance mechanisms of several strains of ticks established from some of the initially reported pyrethroid resistant populations in Mexico. Two of the strains in their study, Coatzacoalcos (Cz) and San Felipe (SF), appeared to have different mechanisms of resistance to pyrethroids. Cz possessed elevated esterase hydrolytic activity against α-and β-naphthyl acetate and permethrin, suggesting metabolic esterases contribute to pyrethroid resistance in this strain. However, in conjunction with earlier results from Miller et al. (1999), SF appeared to possess a target site resistance mechanism. SF was later found to possess a nucleotide substitution in the sodium channel gene coding region leading to a Phe → Ile amino acid substitution in the S6 transmembrane segment of domain III (He et al. 1999). Guerrero et al. (2001) developed a diagnostic polymerase chain reaction (PCR) assay to detect the presence of this nucleotide substitution in individual *B. microplus*, although the nucleotide change was associated with LD_{50} values and not strictly with survival of a pyrethroid application. Hernandez et al. (2000) reported a G → A nucleotide substitution in the sequence of a putative *B. microplus* carboxylesterase-encoding gene, now known as CzEst9 (Jamroz et al. 2000, Pruett et al. 2002), resulting in an Asp → Asn amino acid substitution and creation of an *Eco*RI restriction site. The amino acid substitution occurred at a higher frequency in the Cz strain of *B. microplus* than in the SF strain. We speculated that the mutation might offer enhanced hydrolytic activity and lead to pyrethroid resistance. Hernandez et al. (2002) reported on a diagnostic PCR assay to detect the mu-

This article reports the results of research only. Mention of a proprietary product does not constitute an endorsement or a recommendation by the USDA for its use.

[1] Knipling-Bushland U.S. Livestock Insects Research Laboratory, USDA–ARS, Kerrville, TX 78028.

[2] E-mail: felixg@ktc.com.

[3] Cenid-Parasitología Veterinaria INIFAP-SAGARPA, Apartado Postal 206 Civac, Morelos. CP 62500, Mexico.

tated allele in individual ticks. Our study investigated the association of both the sodium channel and CzEst9 carboxylesterase amino acid substitutions with ticks from the pyrethroid resistant strains Cz and SF. We used the diagnostic PCR assays to individually genotype larvae which survived and which were killed by various concentrations of permethrin in the larval packet test (FAO 1984).

Materials and Methods

Ticks. Rearing of ticks and bioassays were done at the USDA-ARS Cattle Fever Tick Research Laboratory (CFTRL) in Mission, TX, as described by Davey et al. (1980). The sources, establishment and characterization of the metabolic esterase-mediated pyrethroid resistant Cz and the target site insensitivity-mediated pyrethroid resistant SF strain were described by Miller et al. (1999). Bioassays were performed on generation F_{36} and F_{15} of the Cz and SF strains, respectively using the FAO standard larval packet test (FAO 1984). Probit analysis, including probit transformation of percentage mortality and natural logarithm transformation of dose, was performed using the Polo-PC Program (LeOra Software 1987). Pearson chi-square tests on categorical data were performed using the JMP statistical discovery software (SAS Institute 2000). Resistance ratios were determined relative to bioassay data from the reference Gonzalez strain F_{15} generation. To separate bioassay "survivors" and "dead" larvae in resistant strains, bioassays were conducted on generation F_{36} and F_{28} of the Cz and SF strains, respectively. Five concentrations of permethrin were tested in each strain, and each concentration was repeated three times. When bioassays were read, "survivors" and "dead" larvae at each concentration were separated with vacuum and immediately frozen at −70°C for molecular assays.

Nucleic Acids. DNA was purified from individual tick larvae as previously described (Guerrero et al. 2001). Briefly, pooled larvae were frozen in plastic vials placed in dry ice or liquid nitrogen and stored frozen until analyzed. Individual larvae were transferred onto a petri plate on dry ice and placed individually into prechilled 1.5 ml microcentrifuge tubes also kept on dry ice. Twenty microliters of sample buffer (100 mM Tris, pH 8.3; 500 mM KCl) was added to the tube and a disposable pellet pestle for 1.5 ml centrifuge tubes (Kontes, Vineland, NJ) used to crush and grind the larva against the tube wall for ≈20 s, until visual inspection ensured that the larva was broken into several fragments. The tube was transferred back to dry ice until a set of larvae was prepared. The tube contents were briefly microcentrifuged to ensure the liquid and crushed larva were at the tube bottom and placed in a boiling water bath for 3 min. After cooling, 1 μl of this DNA solution was used for PCR.

PCR Conditions. To genotype each tick for the presence of the Phe → Ile substitution in the sodium channel's S6 transmembrane segment of domain III (He et al. 1999), PCR was performed as reported by Guerrero et al. (2001) in thin-walled 0.5 ml microcentrifuge tubes (Bio-Rad, Hercules, CA) using 20-μl reactions optimized for primer annealing temperature and sequence and $MgCl_2$ concentration. Final optimized reaction conditions used 1 μl of genomic DNA solution from a single tick larva, 20 pmol of each primer, 10 mM Tris(hydroxymethyl)aminomethane hydrochloride pH = 8.3, 50 mM KCl, 0.05 mM each dNTP, 1.75 mM $MgCl_2$, and 0.1 μl of a 1:1 vol:vol mix of *AmpliTaq* DNA polymerase (5 U/μl stock; Perkin-Elmer, Foster City, CA), and TaqStart antibody (1.1 μg/μl stock; Clontech, Palo Alto, CA). Amplification was carried out using a DNA engine (MJ Research, Watertown, MA) programmed for 96°C for 2 min followed by 37 cycles, each consisting of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The program also included a final extension step at 72°C for 7 min. Reaction products were fractionated on 2.5% NuSieve agarose (BioWhittaker Molecular Applications, Rockland, ME) TBE gels and DNA visualized by staining with GelStar DNA staining dye (BioWhittaker Molecular Applications) and UV illumination.

To genotype each larvae for the presence of the Asp→Asn substitution in CzEst9, the method described in Hernandez et al. (2002) was used. Primers were derived from the sequence of CzEst9 (clone 13 of Hernandez et al. 2000). The forward primer, GS138B, sequence was 5′-AGCATCGACCTCTC-GTCCAAC-3′ (clone 13 nucleotides 820–840) while the reverse primer, GS139R, sequence was 5′-GTCG-GCATACTTGTCTTCGATG-3′ (clone 13 nucleotides 1170–1191). Amplification of *B. microplus* genomic DNA using these two primers results in a 372-bp product including the region containing the *Eco*RI polymorphism at nucleotide 1120 (Hernandez et al. 2000). Reaction conditions used 1 μl of genomic DNA solution from a single tick larva, four pmol of each primer, 10 mM Tris(hydroxymethyl)aminomethane hydrochloride pH = 8.3, 50 mM KCl, 0.2 mM each dNTP, 3.5 mM $MgCl_2$, and 0.4 μl of a 1:1 vol:vol mix of *AmpliTaq* DNA polymerase (5 U/μl stock; Perkin-Elmer), and TaqStart Antibody (1.1 μg/μl stock; Clontech). Amplification was carried out using a DNA Engine (MJ Research) programmed for 95°C for 2 min followed by 35 cycles, each consisting of denaturation at 94°C for 30 s, annealing at 64°C for 30 s, and extension at 72°C for 1 min. The program also included a final extension step at 72°C for 7 min. PCR products were restriction digested with *Eco*RI, separated by electrophoresis in a 3% NuSieve agarose gel (BioWhittaker Molecular Applications) in TBE buffer (Tris 45 mM, borate 45 mM, EDTA one mM, pH 8.0), and visualized by staining with GelStar.

Results

Bioassays. Table 1 shows the results from the bioassays, verifying previous research which found moderately high pyrethroid resistance in the Cz strain and high pyrethroid resistance in the SF strain (Jamroz et al. 2000). Although only five concentrations were used in the analyses of the Cz F_{36} and SF F_{28} generations,

Table 1. Permethrin bioassay data for Cz and SF strains of *B. microplus*

Strain	Dosage[a] % (AI)	Larvae[b] no.	Alive[c] %	Dead[c] %	LC50 (95% CL) %AI	Slope (±SE)	χ^2	RR[d]
Cz F_{36}	15	267	5	95				
	7.5	242	8	92				
	3.75	294	15	85				
	1.88	230	31	69				
	0.00	98	90	10				
	Generation F_{36} bioassay				0.87 (0.34–1.40)	1.4 (±0.17)	16.49*	67
SF F_{28}	30	437	46	54				
	15	367	62	38				
	7.5	345	77	23				
	3.75	292	80	20				
	0.00	118	100	0				
	Generation F_{15} bioassay				25 (19–39)	1.4 (±0.16)	17.66*	1,923
G	Generation F_{15} bioassay				0.013 (0.012–0.013)	3.72 (±0.25)	14.27*	—

* Indicates significance ($P < 0.05$).
[a] Active ingredient permethrin.
[b] Total number of larvae from all replicates.
[c] Mean of three replicates except 0.00 dose which is only one data point.
[d] R factor, resistance factor = test LC_{50} ÷ Gonzalez reference strain LC_{50}.

the bioassay results verified that the resistance levels were similar to the levels of the Cz F_{22} and SF F_{15} generations which had been bioassayed by Miller et al. (1999) with a more complete range of permethrin doses. The resistance level of SF was so high that it was difficult to prepare permethrin solutions for the larval packet tests which would kill over 50% of the larvae and the resistance level of the SF F_{28} generation could only be estimated. A 30% permethrin dose, which is near the limits that could be formulated in our larval packet tests, killed only 54% of SF larvae. A 15% permethrin dose killed 38% of the SF larvae and 95% of the Cz larvae. Conversely, a 3.75% dose killed 20% of the SF larvae and 85% of the Cz larvae.

PCR Evaluations of Tick Strains. Table 2 shows the results of PCR assays of both SF and Cz larvae which survived or were killed by various doses of permethrin. The Cz strain is characterized by sodium channel alleles fairly evenly distributed between the homozygous susceptible (SS), homozygous resistant (RR) and heterozygotes (SR), as there were 32, 44, and 22% of the SS, SR and RR sodium channel alleles, respectively, in the untreated sample. The Cz strain was dominated by RR alleles at the CzEst9 locus. Cz contained 8, 28, and 62% of the SS, SR and RR CzEst9 alleles, respectively, in the untreated sample. Conversely, the SF strain was dominated by homozygous resistant genotypes at the sodium channel locus. SF contained 5, 25 and 69% of the SS, SR, and RR sodium channel alleles, respectively, in the untreated sample. At the CzEst9 locus, the genotypes of the SF strain were evenly distributed, as there were 23, 38 and 38% of the SS, SR, and RR CzEst9 alleles, respectively, in the untreated sample.

Table 2. PCR diagnosis of sodium channel and CzEst9 alleles in Cz and SF strains of *B. microplus*

Strain	Dosage[a] %(AI)		Larvae no.	*NaChan-CzEst9* genotype[b] (%)								
				SS-SS	SS-SR	SS-RR	SR-SS	SR-SR	SR-RR	RR-SS	RR-SR	RR-RR
Cz	15	Alive	16	0	0	0	0	0	0	0	12	88
		Dead	28	0	4	26	4	18	37	0	4	7
	7.5	Alive	16	0	0	0	0	0	0	19	6	75
		Dead	28	0	11	21	0	18	39	0	11	0
	3.75	Alive	27	0	4	0	0	0	11	0	7	78
		Dead	28	4	14	18	0	14	43	0	4	4
	1.88	Alive	28	0	0	4	0	11	39	0	4	43
		Dead	28	4	7	7	0	18	46	0	7	11
	Untreated		49	2	6	24	2	18	24	4	4	14
SF	30	Alive	28	0	0	0	0	0	0	21	61	18
		Dead	28	0	11	0	4	25	32	7	21	0
	15	Alive	28	0	0	0	0	4	7	11	50	29
		Dead	28	4	4	11	14	32	25	0	11	0
	7.5	Alive	28	0	0	0	7	7	18	0	57	11
		Dead	28	0	11	25	0	29	32	0	0	4
	3.75	Alive	28	4	4	0	7	18	21	4	32	11
		Dead	28	7	39	25	4	11	11	0	0	4
	Untreated		55	0	0	5	5	11	9	18	27	24

[a] Active ingredient permethrin.
[b] Percentages of total where S denotes a wild type allele, R denotes a mutated allele with sodium channel genotype indicated on the left of hyphen and CzEst9 genotype on right.

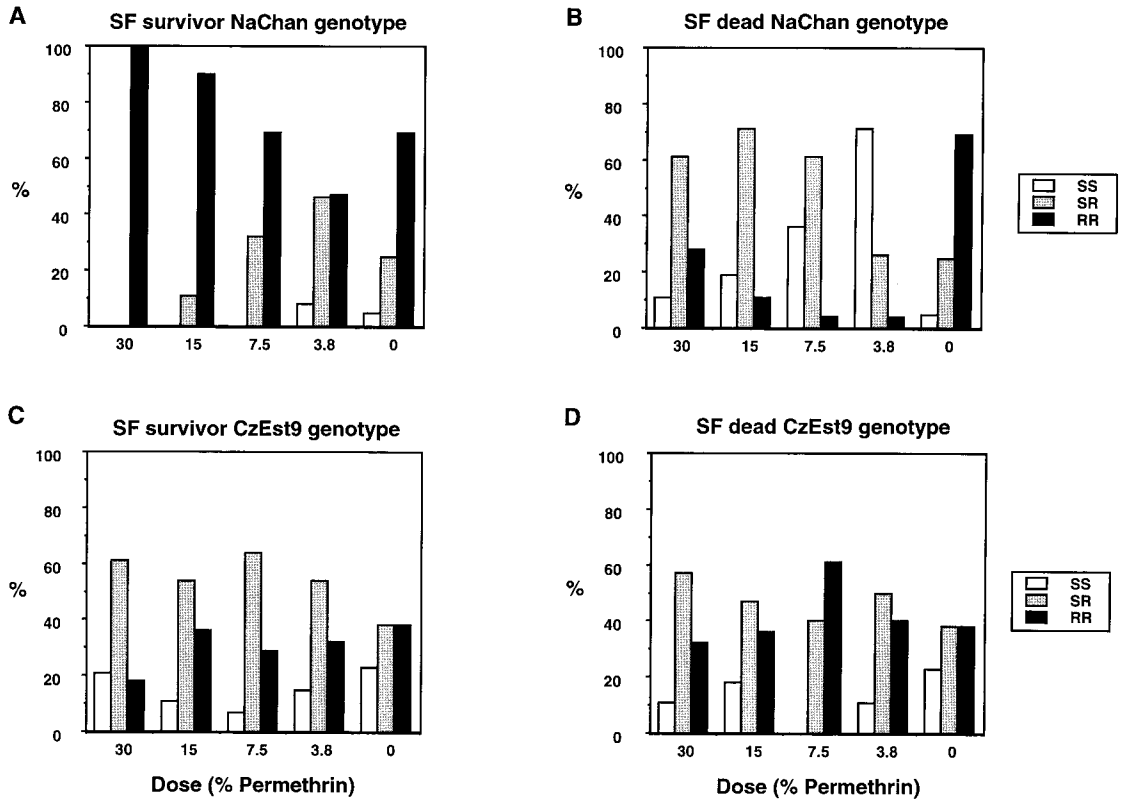

Fig. 1. Genotypes of SF larvae surviving and killed by permethrin. Genomic DNA from SF larvae which survived (A and C) and were killed (B and D) by 30, 15, 7.5 and 3.8% permethrin doses in larval packet bioassays were analyzed by PCR. Both the sodium channel (A and B) and CzEst9 (C and D) genotypes were determined and reported as % homozygous susceptible (SS, open bars), % heterozygotes (SR, stippled bars) and % homozygous resistant (RR, black bars). The 0% permethrin dose shows the genotype distribution for the SF strain in the absence of acaricide.

Figures 1 and 2 show that at the three highest doses for the SF strain and the two highest doses for the Cz strain there were no surviving larvae which did not have at least one copy of the resistant allele of the sodium channel. In fact, examining all the dosage data for both strains, only four of the 199 "alive" larvae possessed the SS sodium channel genotype (Table 2). Of these four "alive" sodium channel "SS" larvae, one was also SS for the CzEst9 genotype, two were heterozygotes and one was RR. Two were from the SF strain (one SS and one SR) and two were from the Cz strain (one SR and one RR). The dead SF larvae had more SS and less RR sodium channel genotypes than the untreated SF group. Thus, the data from both the surviving and killed larvae indicates the RR sodium channel genotype was strongly associated with survival of permethrin treatment. Additionally, the data from both strains showed that as the permethrin dose was increased, a higher sodium channel RR frequency was observed in the surviving larvae (Figs. 1A and 2A).

The SF larvae showed no clear genotypic pattern of survival associated with the CzEst9 genotype. In contrast to the "survivor" results seen with the sodium channel genotype (Fig. 1A), the "survivor" percent RR at the CzEst9 locus (Fig. 1C) does not increase as the permethrin dose is increased. In fact, for each permethrin dose, the percentage of SR survivors was greater than the nontreated group, while the percentage of RR survivors was less than the nontreated group (Fig. 1C). A CzEst9 genotypic pattern associated with permethrin dose is also not apparent with the dead SF larvae. The CzEst9 genotypic pattern in the Cz strain is less straightforward. The permethrin dose effect with the RR sodium channel genotype seen in both strains is absent from the CzEst9 genotype. Higher permethrin doses did not lead to higher CzEst9 RR frequencies among the Cz strain surviving larvae (Fig. 2C). However, at each dose, the CzEst9 RR genotype frequency in the Cz strain survivors is higher than the nontreated Cz strain group. Pearson chi-square tests of significance showed there was a statistically significant association of survival with the CzEst9 RR genotype ($\chi^2 = 5.99$; df = 0.05, 2). More evidence of an effect of the CzEst9 mutation is seen in the Cz strain data of Table 2. At all permethrin doses, there is a consistent shift in favor of survival as the CzEst9 genotype shifts toward more copies of the mutated CzEst9 allele in the progression from RR-SR to the RR-RR genotypes. Tables 3 and 4 show that by pooling

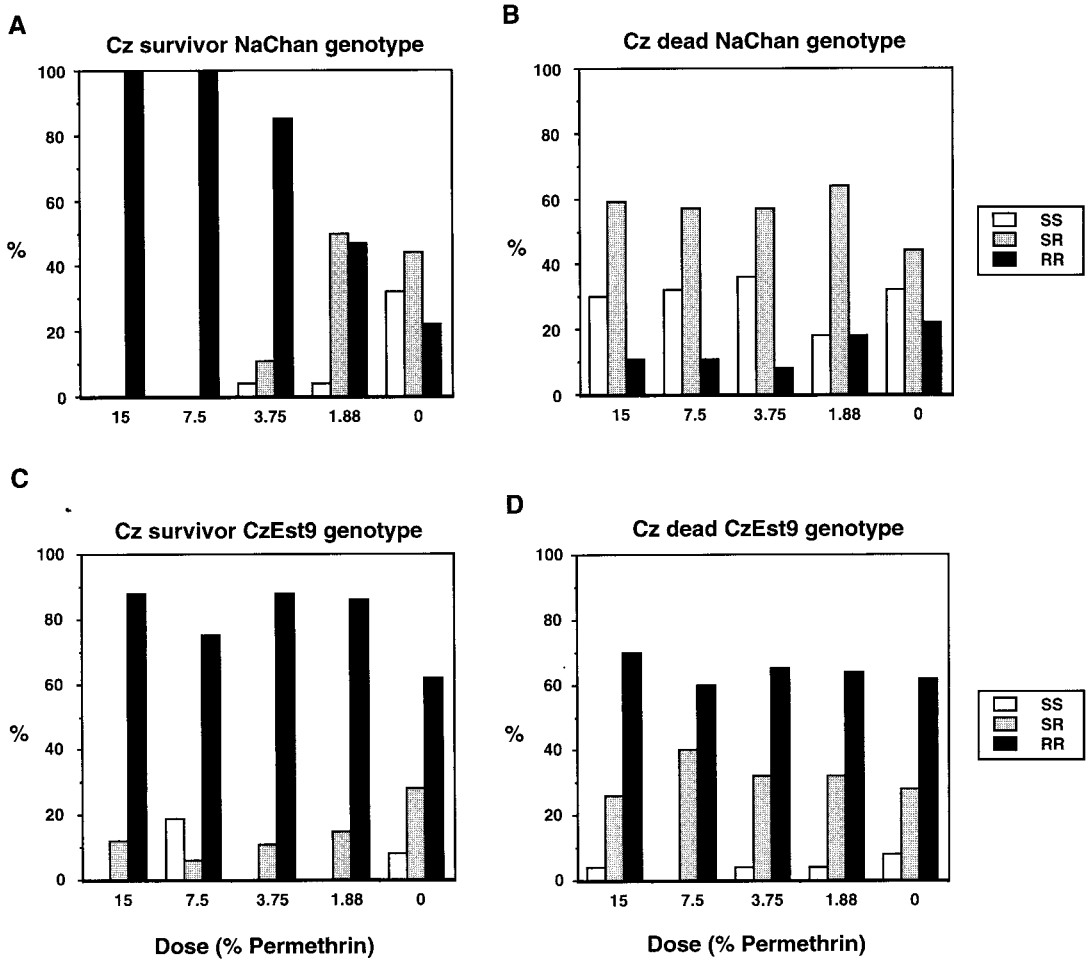

Fig. 2. Genotypes of Cz larvae surviving and killed by permethrin. Genomic DNA from Cz larvae which survived (A and C) and were killed (B and D) by 15, 7.5, 3.8 and 1.88% permethrin doses in larval packet bioassays were analyzed by PCR. Both the sodium channel (A and B) and CzEst9 (C and D) genotypes were determined and reported as % homozygous susceptible (SS, open bars), % heterozygotes (SR, stippled bars) and % homozygous resistant (RR, black bars). The 0% permethrin dose shows the genotype distribution for the Cz strain in the absence of acaricide.

all the larvae by genotype and grouping according to whether the larvae survived or were killed, ignoring strain identity or dosage effects, the sodium channel RR genotype is associated with survival of a permethrin application while the CzEst9 genotype is not.

Discussion

The use of diagnostic PCR coupled with the larval packet assay to separate functionally resistant individual larvae from susceptibles within the SF and Cz strain populations has verified the association of the

Table 3. Mutant sodium channel gene dosage effects on bioassay survival

Genotype[a]	No. larvae[b]	Alive (%)	Dead (%)
SS-SS	6	17	83
SR-SS	11	36	64
RR-SS	15	87	13

[a] S denotes a wild type allele, R denotes a mutated allele with sodium channel genotype indicated on the left of hyphen and CzEst9 genotype on right.
[b] All larvae from each bioassay dose from both SF and Cz strains combined.

Table 4. Mutant CzEst9 gene dosage effects on bioassay survival

Genotype[a]	No. larvae[b]	Alive (%)	Dead (%)
SS-SS	6	17	83
SS-SR	30	7	93
SS-RR	38	3	97

[a] S denotes a wild type allele, R denotes a mutated allele with sodium channel genotype indicated on the left of hyphen and CzEst9 genotype on right.
[b] All larvae from each bioassay dose from both SF and Cz strains combined.

sodium channel domain III S6 segment Phe→Ile amino acid substitution identified by He et al. (1999) with pyrethroid resistance. The PCR data showed that only four of 199 bioassay surviving larvae lacked at least one copy of the mutated sodium channel allele. Although it is not known how these four larvae survived the permethrin treatment, both the SF and Cz strains have a mixed function oxidase component of their permethrin resistance mechanisms (Miller et al. 1999). Perhaps these four larvae survived the permethrin challenge by possessing an elevated oxidase-mediated response.

Despite our data showing a strong association of the domain III sodium channel mutation with resistance, it is possible that an unknown sodium channel mutation is the direct cause of the target site pyrethroid resistance and the association of the domain III S6 Phe→Ile mutation with resistance is coincidental. However, reports by He et al. (1999) and Jamroz et al. (2000) documented thorough screening efforts which failed to detect mutations in the sodium channel open reading frame in the domain II region which contains the classic *kdr* and *super-kdr* mutations from *Musca domestica* (Williamson et al. 1996), *Blattella germanica* (Miyazaki et al. 1996), *Hematobia irritans* (Guerrero et al. 1997) and others. Other pyrethroid resistance-associated sodium channel gene mutations besides *kdr* and *super-kdr* have been reported. Liu et al. (2000) identified four sodium channel open reading frame mutations which are found in highly resistant target site insensitive strains of *B. germanica.* Two mutations are in the intracellular linker connecting domains I and II, a third mutation is in the N-terminus and a fourth mutation is in the C-terminus. However, none of the four have been found in the absence of the *kdr* mutation in S6 of domain II. Pittendrigh et al. (1997) found two mutations in domain III of the *Drosophila melanogaster* sodium channel gene. These mutations, although on separate alleles, were in the analogous positions as the *super-kdr* and *kdr* mutations of domain II and were theorized to function similarly despite being in a different domain. Park et al. (1997) found a mutation in S6 of domain I which conferred pyrethroid resistance to a strain of *Heliothis virescens,* though at a cost of reduced fecundity and mating success for this strain compared with other resistant strains which possessed a mutation analogous to the *kdr* mutation. In fact, the S6 domain I mutation appears to have been replaced by the *kdr*-like mutation in *H. virescens* populations during the 1990s (Zhao et al. 2000). Thus, it appears that target site pyrethroid resistant arthropods which do not possess the classic Leu→Phe *kdr* mutation, possess either a variant resulting amino acid, such as the Leu→His mutation found in *H. virescens* (Park et al. 1997), or mutations in analogous positions of other domains. The tick sodium channel mutation analyzed in our study might function in an analogous fashion to the *kdr* mutation, as both mutations are sited in similar locations of the S6 segments of their respective domains.

Hernandez et al. (2000) found two alleles of a putative carboxylesterase, later identified as CzEst9, whose only amino acid difference was an Asp→Asn substitution. In their analysis of several strains of *B. microplus,* including Cz and SF, the Asp-containing allele was designated as the wild type and the Asn-containing allele designated the mutant. Since the "mutant" Asn allele occurred in all of the larvae from the Cz strain, which possesses a highly active metabolic esterase resistance mechanism (Jamroz et al. 2000), it was postulated this Asp→Asn substitution could have resulted in a highly active esterase which led to pyrethroid resistance through increased metabolic activity. Our results coupled with the increased CzEst9 gene copy number and transcript levels found by Hernandez et al. (2000, 2002) in the Cz strain compared with the other pyrethroid resistant and susceptible strains in their study indicate that the Asp → Asn substitution plays a role in pyrethroid resistance, though perhaps of a lesser importance than the absolute concentration of the CzEst9 gene product, no matter which CzEst9 genotype is present. The Cz strain may attain much of its pyrethroid resistance through increased production of CzEst9 and its associated permethrin-hydrolyzing capability (Pruett et al. 2002). Hernandez et al. (2002) showed the CzEst9 transcript was much more abundant in the Cz strain while barely detectable in two pyrethroid susceptible strains and one target site pyrethroid resistant strain of *B. microplus.* The CzEst9 copy number was also elevated in the Cz strain compared with the susceptible and target site resistant strains (Hernandez et al. 2000). Jamroz et al. (2000) had analyzed the same four strains and shown that the CzEst9 esterase activity was very high in Cz and undetectable in the other three strains. A true measure of the comparative esterase and permethrin hydrolytic activity awaits the expression of active forms of both the Asp- and Asn-containing forms of CzEst9. Although we found a statistically significant association of the "mutant" CzEst9 allele with permethrin resistance, the lack of a pronounced permethrin dose–response on the percentage of CzEst9 RR alleles (homozygous for the "mutation") in surviving larvae is suggestive that CzEst9 R alleles are capable of providing only a limited amount of additional resistance to permethrin beyond that provided by wild type CzEst9 alleles. This is unlike the high level of resistance provided by sodium channel R alleles which have direct impacts upon the effects pyrethroids have on sodium channel properties.

Acknowledgments

We thank Mary Brumley for assistance with the PCR assays and Laurence D. Krska for technical assistance during the bioassays.

References Cited

Davey, R. B., J. Garza, Jr., G. D. Thompson, and R. O. Drummond. 1980. Ovipositional biology of the cattle tick, *Boophilus annulatus* (Acari: Ixodidae), in the laboratory. J. Med. Entomol. 17: 287–289.

FAO. 1984. Acaricide resistance, pp. 246–299. *In* Ticks and tick-borne disease control. A practical field manual, vol. 1. Tick control. FAO, Rome.

Fragoso, S. H., C. N. Soberanes, E. M. Ortiz, V. M. Santamaria, and N. A. Ortiz. 1995. Epidemiology of Ixodicide resistance in the *Boophilus microplus* tick in Mexico, pp. 45–57. *In* III Seminario Internacional de Parasitologia Animal, 11–13 October 1995, Acapulco, Guerrero, Mexico.

Graham, O. H., and J. L. Hourrigan. 1977. Eradication programs for the arthropod parasites of livestock. J. Med Entomol. 13: 629–658.

Guerrero, F. D., R. C. Jamroz, D. Kammlah, and S. E. Kunz. 1997. Toxicological and molecular biological characterization of pyrethroid-resistant horn flies, *Haematobia irritans:* Identification of *kdr* and *super-kdr* point mutations. Insect Biochem. Mol. Biol. 27: 745–755.

Guerrero, F. D., R. B. Davey, and R. J. Miller. 2001. Use of an allele-specific polymerase chain reaction assay to genotype pyrethroid resistant strains of *Boophilus microplus* (Acari: Ixodidae). J. Med Entomol. 38: 44–50.

He, H., A. C. Chen, R. B. Davey, G. W. Ivie, and J. E. George. 1999. Identification of a point mutation in the *para*-type sodium channel gene from a pyrethroid-resistant cattle tick. Biochem. Biophys. Res. Com. 261: 558–561.

Hernandez, R., H. He, A. C. Chen, S. D. Waghela, G. W. Ivie, J. E. George, and G. G. Wagner. 2000. Identification of a point mutation in an esterase gene in different populations of the southern cattle tick, *Boophilus microplus.* Insect Biochem. Mol. Biol. 30: 969–977.

Hernandez, R., F. D. Guerrero, J. E. George, and G. G. Wagner. 2002. Allele frequency and gene expression of a putative carboxylesterase-encoding gene in a pyrethroid resistant strain of the tick, *Boophilus microplus.* Insect Biochem. Mol. Biol.

Jamroz, R. C., F. D. Guerrero, J. H. Pruett, D. D. Oehler, and R. J. Miller. 2000. Molecular and biochemical survey of acaricide resistance mechanisms in larvae from Mexican strains of the southern cattle tick, *Boophilus microplus.* J. Insect Physiol. 46: 685–695.

LeOra Software. 1987. A user's guide to probit or logit analysis. LeOra Software, Berkeley, CA.

Liu, Z., S. M. Valles, and K. Dong. 2000. Novel point mutations in the German cockroach *para* sodium channel gene are associated with knockdown resistance (*kdr*) to pyrethroid insecticides. Insect Biochem. Mol. Biol. 30: 991–997.

Miller, R. J., R. B. Davey, and J. E. George. 1999. Characterization of pyrethroid resistance and susceptibility to coumaphos in Mexican *Boophilus microplus* (Acari: Ixodidae). J. Med. Entomol. 36: 533–538.

Miyazaki, M., K. Ohyama, D. Y. Dunlap, and F. Matsumura. 1996. Cloning and sequencing of the *para*-type sodium channel gene from susceptible and *kdr*-resistant German cockroaches (*Blattella germanica*) and house fly (*Musca domestica*). Mol. Gen. Genet. 252: 61–68.

Park, Y., M.F.J. Taylor, and R. Feyereisen. 1997. A valine421 to methionine mutation in IS6 of the *hscp* voltage-gated sodium channel associated with pyrethroid resistance in *Heliothis virescens* F. Biochem. Biophys. Res. Com. 239: 688–691.

Pittendrigh, B., R. Reenan, R. H. ffrench-Constant, and B. Ganetzky. 1997. Point mutations in the *Drosophila* sodium channel gene *para* associated with resistance to DDT and pyrethroid insecticides. Mol. Gen. Genet. 256: 602–610.

Pruett, J. H., F. D. Guerrero, and R. Hernandez. 2002. Isolation and identification of an esterase from a Mexican strain of *Boophilus microplus* (Acari: Ixodidae). J. Econ. Entomol.

SAS Institute. 2000. JMP statistics and graphics guide. SAS Institute, Cary, NC.

Williamson, M. S., D. Martinez-Torres, C. A. Hick, and A. L. Devonshire. 1996. Identification of mutations in the housefly *para*-type sodium channel gene associated with knockdown resistance (*kdr*) to pyrethroid insecticides. Mol. Gen. Genet. 252: 51–60.

Zhao, Y., Y. Park, and M. E. Adams. 2000. Functional and evolutionary consequences of pyrethroid resistance mutations in S6 transmembrane segments of a voltage-gated sodium channel. Biochem. Biophys. Res. Com. 278: 516–521.

Received for publication 27 November 2001; accepted 17 May 2002.